
RNase H cleavage of RNA hybridized to oligonucleotides containing methylphosphonate, phosphorothioate and phosphodiester bonds

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ABSTRACT

Three types of 14-mer oligonucleotides were hybridized to human β -globin pre-mRNA and the resultant duplexes were tested for susceptibility to cleavage by RNase H from *E. coli* or from HeLa cell nuclear extract. The oligonucleotides contained normal deoxynucleotides, phosphorothioate analogs alternating with normal deoxynucleotides, or one to six methylphosphonate deoxynucleosides. Duplexes formed with deoxyoligonucleotides or phosphorothioate analogs were susceptible to cleavage by RNase H from both sources, whereas a duplex formed with an oligonucleotide containing six methylphosphonate deoxynucleosides alternating with normal deoxynucleotides was resistant. Susceptibility to cleavage by RNase H increased parallel to a reduction in the number of methylphosphonate residues in the oligonucleotide.

Stability of the oligonucleotides in the nuclear extract from HeLa cells was also tested. Whereas deoxyoligonucleotides were rapidly degraded, oligonucleotides containing alternating methylphosphonate residues remained unchanged after 70 minutes of incubation. Other oligonucleotides exhibited intermediate stability.

INTRODUCTION

Antisense oligonucleotides are increasingly used as modulators of cellular and viral gene expression (see ref. 1–4 for review). Three classes of oligonucleotides have been used in recent investigations: antisense deoxyoligonucleotides (D-oligos), their modified counterparts and antisense RNA. All three classes have been effective in inhibiting expression of specific genes. For example, in their pioneering work Zamecnik and Stephenson (5) showed that D-oligos complementary to a segment of reiterated terminal sequence of Rous sarcoma virus inhibit viral replication. More recently phosphorothioate deoxyoligonucleosides (S-oligos, developed by Eckstein and coworkers, see ref. 6 for review), methylphosphonate deoxyoligonucleosides (MP-oligos, developed by Miller, Ts'o and coworkers, reviewed in ref. 7) as well as D-oligos have been shown to inhibit replication of the human immunodeficiency virus when they were complementary to essential viral sequences (8–11). Other modified oligonucleotides that inhibit expression of specific genes include phosphoroamidate oligonucleosides (9), α -oligonucleotides (12,13), and polylysine (14), psoralen (15) and acridine conjugated oligonucleotides (16,17). The discovery that the expression of some procaryotic genes is controlled in vivo by endogenous antisense RNA (reviewed in 18 and 19), and that expression of thymidine kinase can be inhibited by antisense RNA transcribed from a recombinant expression vector (20,21) showed that antisense RNA may also be useful in inhibiting the expression of specific genes. This early work led to a number of subsequent studies extensively reviewed in ref. 18.

Most of the reports discussed above focused on the final effect of antisense oligonucleotides on gene expression without detailed studies of the mechanism of inhibition. The two most likely mechanisms of inhibition appear to be 1) direct blocking in pre-mRNA and/or mRNA of sequences important for processing or translation and 2) degradation of the RNA transcript by RNase H at the site of oligonucleotide binding. RNase H cleaves the RNA component of RNA:DNA hybrids and is abundant in the cytoplasm and nucleus of a large number of organisms (22). Recent reports show that cleavage of RNA:DNA duplexes by RNase H was predominantly responsible for the inhibitory activity of D-oligos in several experimental systems (23–25). However, only limited data are available regarding the mechanism of action of modified oligonucleotides (3,26,27). In addition, discrepancies exist concerning the effects of antisense molecules. For example, antisense MP-oligos were found inhibitory in some but not all systems (10,11, 26,28,29) and a 1000 fold excess of antisense RNA did not inhibit the activity of chloramphenicol acetyl transferase expressed in transfected CV1 cells (30). These observations suggest that more detailed investigations are needed to discern the mechanism of inhibition by antisense oligonucleotides.

Since there are indications that RNA:MP-oligo duplexes are resistant to RNase H (3,26) we decided to study in more detail the effect of incorporation of methylphosphonate deoxynucleosides into D-oligos on the susceptibility of pre-mRNA:DNA duplexes to RNase H cleavage. To this end we have used a series of 14-mer oligonucleotides substituted with one to six methylphosphonate deoxynucleosides. For comparison, we have also tested a 14-mer D-oligo and a 14-mer S-oligo containing alternating deoxynucleotides and phosphorothioate analogs. We found that D-oligo and an alternating S-oligo form duplexes with pre-mRNA that are cleaved by E.coli RNase H and by the RNase H present in a crude nuclear extract from HeLa cells. In contrast, RNA in duplexes formed with an MP-oligo containing six methylphosphonate deoxynucleosides alternating with deoxynucleotides is resistant to cleavage by RNase H from both sources but becomes susceptible if the number of methylphosphonate deoxynucleosides in the oligonucleotide is decreased.

MATERIALS AND METHODS

Oligonucleotide synthesis. Oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems) using standard β -cyanoethyl cycles (31). Materials for synthesis were supplied by Applied Biosystems or American Bionetics. D-oligos were deblocked and cleaved from the column with following the Applied Biosystems protocol. S-oligos were obtained in a purified form from Dr. Scott Eadie (Applied Biosystems). MP-oligos were cleaved from the solid support column by ethylenediamine: absolute ethanol (1:1 v/v) treatment at 55°C for 55 minutes, eluted with absolute ethanol followed by ethanol-water (1:1 v/v), lyophilized and resuspended in water. O.D.₂₆₀ was measured for each sample and the oligonucleotides were used in this form for all experiments. When necessary, all oligonucleotides were end labeled with [³²P] γ -labeled ATP using T4 polynucleotide kinase in 100 mM Tris pH 7.5, 20 mM MgCl₂, 10 mM DTT, 0.2 mM spermidine, 0.2 mM EDTA at 37°C for 30 minutes as recommended by the supplier (New England Biolabs). **Pre-mRNA transcription.** The DNA plasmid containing the human β -globin gene cloned under the control of the SP6 promoter (pSP64Hb Δ 6, see ref. 32), was digested with the restriction endonuclease Bam HI and transcribed and capped in vitro as described (33) using SP6 polymerase and [³²P] labeled GTP (New England Nuclear). This produced a human β -globin pre-mRNA truncated at the 3' end of the second exon.

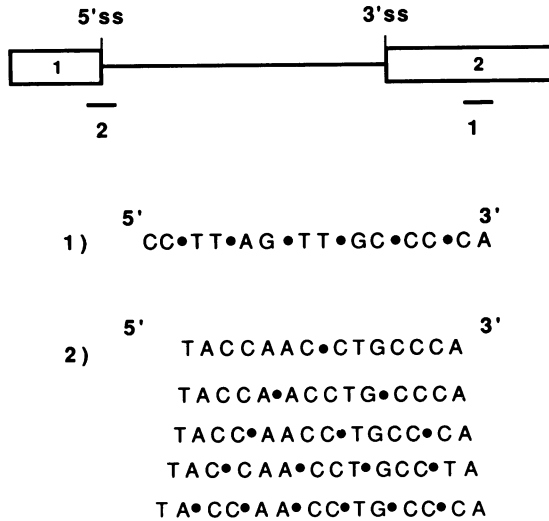


Figure 1. Target RNA and antisense oligonucleotides. The structure of the truncated human β -globin pre-mRNA used for hybridization with the oligonucleotides is shown. Exons (boxes) intron (thin line) and splice sites (5' and 3' ss) are indicated. The transcript is terminated at a Bam HI site close to the 3' end of the second exon. The positions where oligos #1 and #2 hybridize to the pre-mRNA as well as the sequence of the oligos are also shown. Dots in the oligonucleotide sequence show the position of the methylphosphonate or phosphorothioate internucleotide bonds in MP- and S-oligos. Oligo #1 was used as a normal deoxyoligonucleotide (D.1), an alternating phosphorothioate (S.1) and an alternating methylphosphonate (MP.1). Oligo #2 was used as a deoxyoligonucleotide (D.2) or containing an increasing number of methylphosphonate residues per molecule (1MP.2–6MP.2).

RNase H cleavage. E.coli RNase H was obtained from Bethesda Research Laboratories. Nuclear extract from HeLa cells (32,34) was used as a source of eucaryotic RNase H. Oligonucleotides (25 pmoles) and [³²P] labeled pre-mRNA (10 pmoles) were hybridized in vitro at 40°C for 10 minutes in 10 µl of the following reaction mixture: for cleavage by E. coli RNase H, the hybridization was performed in 130 mM ammonium chloride; for cleavage by RNase H from HeLa cell nuclear extract, the hybridization mixture contained 12.5 mM ATP, 8.25 mM MgCl₂, 50 mM creatine phosphate and 6.5% polyvinyl alcohol. Following hybridization, for the E.coli enzyme, the reaction was performed at 37°C for 30 minutes in a total volume of 20 µl containing 130 mM ammonium chloride, 10 mM Tris pH 7.5, 10 mM magnesium acetate, 5% sucrose and 1µl of RNase H. For the HeLa enzyme, 15 µl of the nuclear extract was added and the reaction was performed at 30°C for 15 minutes. The extract contributed several buffer components so that the final concentrations of the reagents were 5 mM ATP, 3.3 mM MgCl₂, 20 mM creatine phosphate, 2.6% polyvinyl alcohol, 12.8 mM HEPES, pH 7.9, 14% glycerol, 60 mM KCl, 0.12 mM EDTA and 0.7 mM DTT, i.e., standard conditions for in vitro splicing of pre-mRNA (34). RNase H cleavage products were analyzed by electrophoresis on a 5% polyacrylamide followed by autoradiography.

Primer extension. Primer extension assay was performed in a 10 μ l reaction containing 100 mM Tris pH 7.5, 100 mM MgCl₂, 200 mM NaCl, 25 pmoles of oligonucleotide, 10 pmoles of [³²P]-pre-mRNA, 5 μ Ci of [³²P]- α -labeled dATP, 2.5 mM deoxynucleotide triphosphates and 1 μ l of AMV reverse transcriptase (Life Sciences). The primer extension

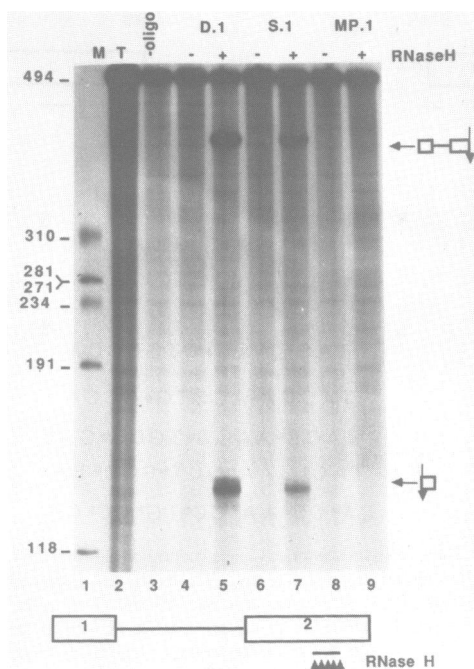


Figure 2 A. Cleavage of pre-mRNA in duplexes with D-oligo, S-oligo, or MP-oligo by RNase H from *E. coli*. [^{32}P]-pre-mRNA was hybridized with oligonucleotide # 1 (see Fig. 1) in the form of D-oligo (D.1, lanes 4 and 5), alternating S-oligo (S.1, lanes 6 and 7), or alternating MP-oligo (MP.1, lanes 8 and 9) and incubated without (-) or with (+) RNase H from *E. coli*. The resulting RNA products were separated on a 5% polyacrylamide sequencing gel. A schematic structure of RNase H cleavage products is shown at right. Below the figure, the structure of the pre-mRNA, the position of binding of oligonucleotide # 1, and cleavage by RNase H are shown. In this and subsequent figures, M (lane 1) denotes size markers (Hae III digest of ΦX174 in this figure and products of β -globin pre-mRNA splicing in the following figures), T (lane 2) denotes untreated RNA transcript (approximately 3 times the amount of radioactive RNA was loaded in this lane), -oligo (lane 3) denotes pre-mRNA mock hybridized and incubated with RNase H in the absence of oligonucleotide.

products were electrophoresed and autoradiographed as above.

Stability of oligonucleotides. Oligonucleotides, 5'-end labeled with [^{32}P], were incubated in the HeLa cell nuclear extract for 0–70 minutes in the same buffer conditions as described above and subsequently analyzed on a 20% polyacrylamide sequencing gel. The gels were autoradiographed and the amount of intact material and degradation products was quantitated by densitometry. As a measure of degradation a ratio of the amount of the intact 14-mer to a smallest degradation product, the mononucleotide, was calculated and plotted in figures 3 and 7. This way of calculation was carried out to compensate for the activity of phosphatases, which might be present in the nuclear extract and could gradually remove the radioactive label.

RESULTS

A capped fragment of human β -globin pre-mRNA (Fig. 1), obtained by transcription in vitro (see Materials and Methods), was hybridized to three types of 14-mer oligonucleotides that included a normal deoxynucleotide (D-oligo), a phosphorothioate analog (S-oligo),

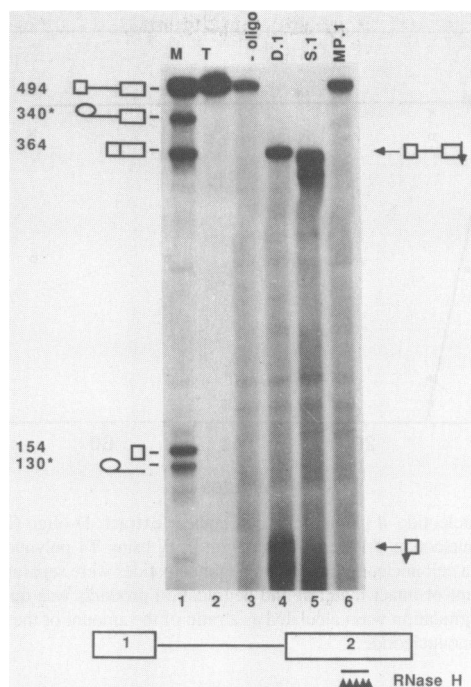


Figure 2B. Cleavage of pre-mRNA in duplexes with D-oligo, S-oligo or MP-oligo by RNase H from HeLa cell extract. The same [32 P]-pre-mRNA:oligo duplexes as in A were treated with RNase H from HeLa cell nuclear extract (lanes 4, 5, and 6). RNA products were analyzed as in A and the structure and position of the RNase H cleavage products are shown on the right. In lane 1, pre-mRNA was spliced in vitro and the resulting products used as size markers. The size and structure of the splicing products is shown at left. Asterisk indicates aberrant migration on the gel of the splicing intermediates containing lariat. Below the figure, a diagram of the RNase H site of cleavage is shown.

and a series of oligonucleotides containing an increasing number of methylphosphonate deoxynucleosides (MP-oligos). The D-oligo, the S-oligo and one of the MP-oligos were complementary to nucleotides 360–373 in the second exon of human β -globin pre-mRNA (Fig. 1, oligo 1). The S-oligo and the MP-oligo contained alternating phosphodiester and modified internucleotide bonds. An additional series of MP-oligos, which contained from one to six methylphosphonate deoxynucleosides positioned in the oligonucleotides as shown in Fig. 1, was complementary to the 5' splice site, at nucleotides 148–161 (Fig. 1, oligo 2). Substitution of six methylphosphonate deoxynucleosides resulted in an MP-oligo containing alternating methylphosphonate and phosphodiester internucleotide bonds. The duplexes formed with these oligonucleotides were tested for their susceptibility to hydrolysis by RNase H.

The pre-mRNA hybridized with the normal D-oligo, complementary to the second exon (Fig. 1, oligo 1), was incubated with E.coli RNase H and the RNA isolated from the reaction was analyzed on a 5% polyacrylamide sequencing gel. Part of the RNA was cleaved into two fragments of approximately 360 and 120 nucleotides (Fig. 2A, lane 5). Longer incubation with a larger amount of enzyme led to complete cleavage of the RNA into the same two fragments (results not shown, see also Fig. 5, lane 9). The larger cleavage product

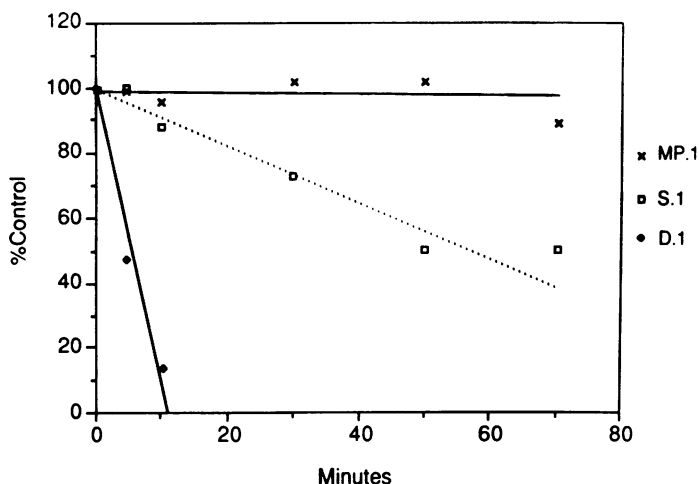


Figure 3. Stability of oligonucleotide #1 in a HeLa cell nuclear extract. D-oligo (D.1), S-oligo (S.1) and MP-oligo (MP.1) forms of oligonucleotide #1 were labeled with [32 P] using T4 polynucleotide kinase and incubated from 0–70 minutes in a HeLa cell nuclear extract. The oligonucleotides were separated on a 20% polyacrylamide sequencing gel and the amount of intact material and degradation products was quantitated by densitometry of autoradiograms. Extent of degradation was calculated as a ratio of the amount of the intact 14-mer to the smallest degradation product, the mononucleotide.

represents a capped 5' fragment of the pre-mRNA located upstream from the binding site of the oligonucleotide while the smaller fragment represents a 3' part of the RNA transcript. The mobility of the fragments on the gel is in agreement with their size predicted from the RNA sequence data. The cleavage reaction required both the oligonucleotide and the enzyme, since the RNA remained intact in control samples missing either of these components, (Fig. 2A, lanes 1 and 2, respectively). Similarly, oligonucleotide and enzyme-dependent cleavage by RNase H was also observed for a duplex containing the alternating S-oligo (Fig. 2A, lane 7) although densitometry of the film showed that the yield of the generated fragments was approximately 20 % lower than that for the D-oligo containing duplex (compare lanes 5 and 7 in Fig. 2A). In contrast, RNA hybridized under the same conditions with alternating MP-oligo was resistant to cleavage by RNase H (Fig. 2A, lane 9).

To determine whether eucaryotic RNase H would cleave these duplexes, the same samples were incubated with a nuclear extract from HeLa cells known to contain high levels of RNase H activity (32). The crude extract was used because it more closely resembles the intracellular conditions to which the duplexes would be exposed in vivo. Similarly as in the previous experiment, pre-mRNA in duplexes with either D-oligo or S-oligo was hydrolyzed by RNase H in the extract (Fig. 2B, lanes 4 and 5, respectively) whereas the pre-mRNA hybridized with MP-oligo remained intact (Fig. 2B, lane 6). The 360 nucleotide fragment generated by RNase H cleavage was stable in the extract and is clearly visible on a gel but the shorter 3' fragment, which was not capped, was largely degraded. The instability of the latter fragment is in agreement with the observations that the nuclear extract from HeLa cells contains a 5' to 3' exonuclease activity which is inhibited by the cap structure at the 5' end of the pre-mRNA transcript (32 and our unpublished observations).

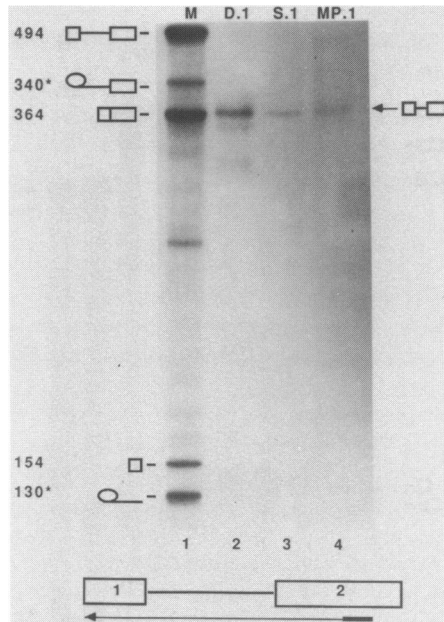


Figure 4. Primer extension analysis of oligonucleotide analogues. D-oligo, S-oligo, and MP-oligo forms of oligonucleotide #1 were hybridized to the pre-mRNA, extended with AMV reverse transcriptase and the products separated on a 5% polyacrylamide gel. The structures and positions of the primer extension products for D-oligo (D.1, lane 2), S-oligo (S.1, lane 3), and MP-oligo (MP.1, lane 4) are shown on the right. A diagram of the primer extension reaction is shown at the bottom.

The above results showed that pre-mRNA hybridized with D-oligo and an alternating S-oligo formed duplexes that were substrates for RNase H. However, the lack of cleavage of the pre-mRNA hybridized with an alternating MP-oligo either by *E. coli* RNase H or by the HeLa cell nuclear extract could have several possible explanations. Although it is likely that the pre-mRNA:MP-oligo duplex may not be a substrate for RNase H, it is also possible that 1) the alternating MP-oligo is rapidly degraded, especially in the crude nuclear extract, 2) it does not hybridize to pre-mRNA under the conditions of the experiment, or 3) it directly inhibits RNase H. A series of experiments have been performed to distinguish between these possibilities.

To test the stability of the MP-oligo in the extract, the oligo was labeled with [^{32}P] using T4 polynucleotide kinase, incubated in the nuclear extract and analyzed on a 20% polyacrylamide sequencing gel. The autoradiograms of the gel were quantitated by densitometry (see Materials and Methods). For comparison, the stabilities of D-oligo and S-oligo were also tested. Of the three oligonucleotides, MP-oligo was the most stable and remained essentially intact for at least 70 minutes of incubation. Degradation of S-oligo was also slow, with approximately 50% of the material remaining at the end of incubation whereas D-oligo was almost completely degraded after 10 minutes (Fig. 3).

To determine whether the MP-oligo is able to hybridize to the pre-mRNA, we tested it in a primer extension assay (Fig. 4). All three oligonucleotides, including MP-oligo, generated extension products of the expected length (374 nucleotides) indicating that,

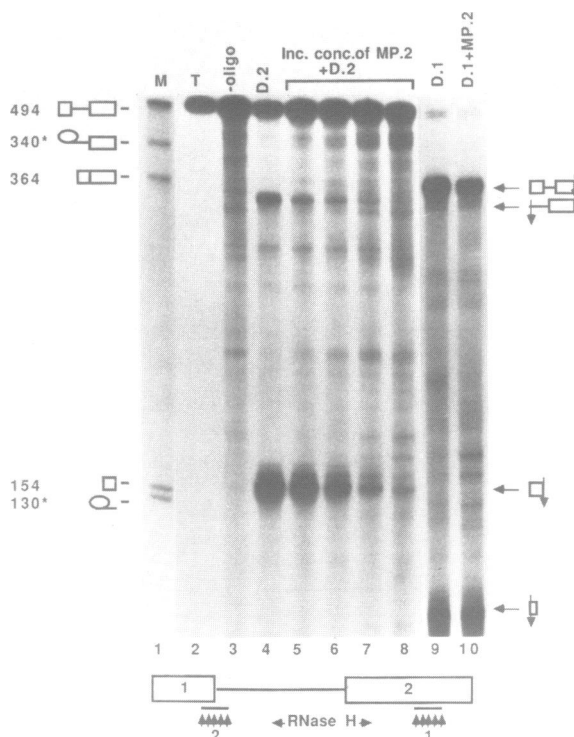


Figure 5. Competition assay using D-oligo #2, alternating MP-oligo #2 and D-oligo #1. 10 pmoles of [32 P]-labeled pre-mRNA was hybridized with 0, 0.002, 0.02, 0.2 or 10 O.D.₂₆₀ of MP-oligo #2 followed by hybridization with 0.002 O.D.₂₆₀ of D-oligo #2 and treatment with RNase H from *E. coli* (lanes 4–8, respectively). The position and structure of the RNase H cleavage products after separation on a 5% polyacrylamide gel are shown by the middle two diagrams on the right. To test for direct inhibition of RNase H by MP-oligo, pre-mRNA was hybridized without (lane 9) or with (lane 10) MP-oligo #2 followed by hybridization with D-oligo #1 and treatment with RNase H from *E. coli*. The position and structure of these RNase H cleavage products are shown by the top and bottom diagrams on the right. Below the figure is a schematic representation of the RNase H cleavage site for the oligonucleotides used above.

similarly to D-oligo and S-oligo, MP-oligo formed a duplex with the pre-mRNA. Additional faint bands seen in lane 2 result from premature termination of reverse transcription. The lower yield of extension products from the S-oligo and MP-oligo duplexes as compared to the D-oligo duplex is probably due to the lower efficiency of the AMV reverse transcriptase with the modified primers, as previously observed (35). Since S-oligo and MP-oligo yielded similar amounts of extension product, the resistance of the RNA:MP-oligo duplex to hydrolysis by RNase H (Fig. 2A, lane 9 and Fig. 2B, lane 6) was not due to the lack of hybridization of this oligonucleotide to the pre-mRNA.

To obtain additional evidence that the interaction of MP-oligo with the pre-mRNA is sequence specific and to show that the MP-oligo does not directly inhibit RNase H, we carried out a competition experiment with different D- and MP-oligos. The pre-mRNA was hybridized to increasing concentrations of the alternating MP-oligo complementary to the 5' splice site (see Fig. 1B, oligo 2) followed by incubation with a normal D-oligo

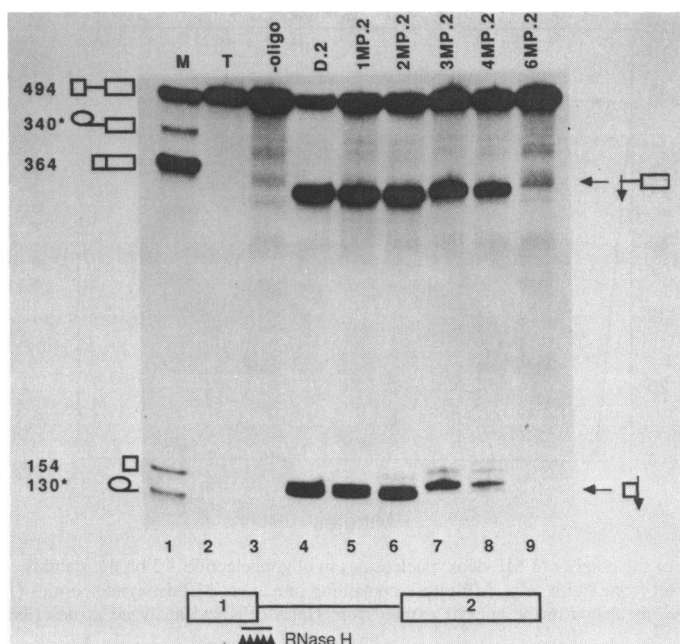


Figure 6. Effect of the number of MP-deoxynucleosides in oligonucleotide #2 on the susceptibility of RNA:MP-oligo duplexes to RNase H cleavage. Pre-mRNA:oligo duplexes were formed and treated with *E. coli* RNase H as described in Materials and Methods and analyzed on a 5% polyacrylamide sequencing gel. Size markers and controls (lanes 1–3) are as described in Fig. 2A. Lane 4, RNase H cleavage of a duplex with D-oligo #2 (D.2). Lanes 5–9, RNase H cleavage of duplexes with MP-oligos containing one to six MP-deoxynucleosides, respectively. Diagrams are as described in Fig. 2A.

of the same sequence. The duplexes were then treated with RNase H in the nuclear extract as described above. As expected, in the absence of MP-oligo, the pre-mRNA was cleaved into two RNase H cleavage products, approximately 330 and 150 nucleotides long (Fig. 5, lane 4). Cleavage with RNase H was progressively inhibited by increasing amounts of MP-oligo as indicated by the disappearance of the cleavage products in lanes 5–8. Other bands visible in these lanes represent unspecific degradation products since they are also present in the control sample incubated without either oligonucleotide (Fig. 5, lane 3). To ascertain that the MP-oligo did not inhibit RNase H directly, the D-oligo complementary to the exon sequence (Fig. 1, oligo 1) was hybridized to the pre-mRNA with or without the alternating MP-oligo complementary to the 5' splice site (Fig. 1, oligo 2). When the duplexes were incubated with the nuclear extract, RNase H cleaved the pre-mRNA:D-oligo duplex and was not inhibited in the presence of the MP-oligo (Fig. 5, lanes 9 and 10).

We conclude from the experiments that 1) the MP-oligo did not have any direct inhibitory effect on RNase H; 2) the increasing resistance of duplexes seen in lanes 5–8 must be due to competition between D-oligo and MP-oligo for the same sequence in the pre-mRNA; 3) the MP-oligo formed a sequence specific duplex with the pre-mRNA which was resistant to RNase H.

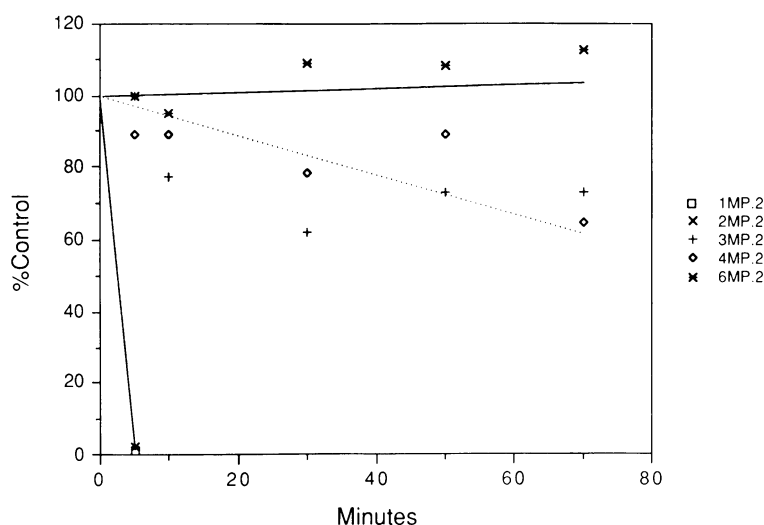


Figure 7. Effect of the number of MP-deoxynucleosides in oligonucleotide #2 on the stability of the MP-oligos in a nuclear extract from HeLa cells. MP-oligos containing one to six MP-deoxynucleosides (1MP.2–6MP.2, respectively) were incubated in the nuclear extract from HeLa cells and analyzed as described in Fig.3.

Since the above results showed that duplexes between pre-mRNA and alternating MP-oligo are resistant to RNase H we wanted determine the minimum number of methylphosphonate deoxynucleosides required to confer resistance to a duplex molecule. We have synthesized a series of 14-mer oligonucleotides, containing from one to six methylphosphonate deoxynucleosides positioned in the oligonucleotides as shown in Fig. 1 (Fig. 1, oligo 2). To ascertain that the resistance to RNase H observed above is not due to a particular sequence or secondary structure at the oligonucleotide binding site in the second exon, this series was made complementary to a different region of the pre-mRNA, at the 5' splice site. These MP-oligos were hybridized to the pre-mRNA and the duplexes were subjected to hydrolysis by *E. coli* RNase H.

RNA hybridized to MP-oligos containing one or two methylphosphonate deoxynucleosides was cleaved by RNase H almost as easily as that in the control duplex with D-oligo (Fig. 6, compare lane 4 with lanes 5 and 6). RNA in duplexes with MP-oligos which contained three, four and six methylphosphonate deoxynucleosides, i.e., in which methylphosphonate bonds were separated by three, two or one phosphodiester bond (see Fig. 1, oligo 2), was increasingly resistant to cleavage by the enzyme (Fig. 6, lanes 7–9, respectively). Interestingly, the resistance of these duplexes to RNase H hydrolysis paralleled the stability of the corresponding MP-oligos in the nuclear extract. MP-oligos containing one or two methylphosphonate deoxynucleosides were degraded rapidly and the stability of the oligonucleotides increased with an increased number of methylphosphonate deoxynucleosides in the molecule (Fig. 7)

DISCUSSION

Results presented above show that D-oligo, S-oligo and MP-oligos hybridize to pre-mRNA in a sequence specific manner. Duplexes formed with the first two types of oligonucleotides are susceptible to cleavage by RNase H from *E. coli* and from HeLa cells. This agrees

with the results of Stein et al. (27) on cleavage of polyA:phosphorothioate oligo dT duplexes by RNase H from *E. coli* and with the reports of other groups on inhibition of translation by RNase H cleavage of D-oligo containing duplexes (23–25). In contrast, duplexes formed with alternating MP-oligos are not substrates for RNase H from *E. coli* or HeLa cells. Their resistance to the enzyme decreases with the decrease in the number of methylphosphonate deoxynucleosides in the MP-oligo. Our results also show that the presence of phosphorothioate and methylphosphonate deoxynucleosides promotes the stability of the oligonucleotides in the nuclear extract from HeLa cells.

It has been shown by a number of investigators that D-oligos, S-oligos and MP-oligos can be used *in vivo* to inhibit expression of a specific gene in a sequence specific manner (reviewed in 1–4). The results presented here suggest different mechanisms of inhibition by different classes of oligonucleotides. Whereas D-oligos and S-oligos probably lead to the degradation of target RNA transcript by RNase H, MP-oligos containing only methylphosphonate deoxynucleosides or alternating MP-oligos exert their effect in a different manner, most likely by interfering with the accessibility of RNA to factors essential for processing, transport or translation. In most experiments, designed to eliminate RNA viruses, viral transcripts, or the inappropriate expression of endogenous RNA, the cleavage of an RNA:DNA hybrid is desired. In some situations, however, the lack of cleavage of the RNA can be advantageous. For example, the ability to block specific sequences in intact pre-mRNA may be very useful in studies of the interactions of the splicing factors with the pre-mRNA substrate. In fact, our recent experiments show that alternating MP-oligo complementary to the 5' splice site of the β -globin pre-mRNA will hybridize to the pre-mRNA substrate without degrading it and will inhibit splicing in the nuclear extract from HeLa cells (Furdon and Kole, unpublished).

Our results with MP-oligos containing an increasing number of methylphosphonate residues suggest that the properties of the MP-oligos may be manipulated to achieve a desired effect. Introduction of a few methylphosphonate deoxynucleosides will not inhibit the activity of RNase H but will markedly increase the stability of the resulting MP-oligos. Thus, if degradation of the target RNA is the goal of the experiment such MP-oligos should be more effective than normal D-oligos. Conversely, for blocking of specific sequences in intact RNA, MP-oligos made exclusively with methylphosphonate deoxynucleosides or with alternating methylphosphonate and normal deoxynucleotides should be preferable. The alternating MP-oligos have a higher negative charge than fully modified MP-oligos allowing for easier handling and purification on polyacrylamide gels. In addition, in alternating MP-oligos the number of diastereoisomers generated during chemical synthesis (36) is drastically reduced which may increase the effective concentration of the molecules able to hybridize to target RNA. Finally, MP-oligos that contain two phosphodiester bonds surrounded by stretches of methylphosphonate deoxynucleosides can be used as tools for precise cleavage of the target RNA by RNase H.

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